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I, the undersigned, being an officer duly authorized in accordance with the provision of the Patent Act, 1970 hereby certify that annexed hereto is the true copy of the ***Application, Provisional*** *and* ***Complete Specification*** *and* ***Drawing Sheets*** *filed in connection with Application for Patent No.* ***1274/Del/02*** *dated* ***18th December 2002.***

Witness my hand this 17th Day of September 2003.

(S.K. PANGASA)
Assistant Controller of Patents & Designs

PRIORITY DOCUMENT
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1270-02

FORM-1

(To be filed in Triplicate)

THE PATENTS ACT, 1970

18 DEC 2002

(39 of 1970)

APPLICATION FOR GRANT OF A PATENT

[See Sections 5(2) 7, 54 and 135)

1. We 1) DEPARTMENT OF BIOTECHNOLOGY, of Block 2, 7th Floor, CGO Complex, Lodi Road, New Delhi-110 003; and
2) ALL INDIA INSTITUTE OF MEDICAL SCIENCES, of Ansari Nagar, New Delhi-110 029.

2. hereby declare-

(a) that ~~I am~~ / We are in possessin of an Invention titled

Title: THE CHARACTERIZATION OF HUP B GENE ENCODING HISTONE LIKE PROTEIN OF MYCOBACTERIAL TUBERCULOSIS

(b) that the Provisional / ~~Complete~~ Specification relating to this invention filed with this application.

DUPLICATE

(c) that there is no lawful ground of objection to the grant of a patent to me / us.

3. Further declare that the inventor(s) for the said invention is / are :

Surname first and then name of inventor/s: 1) HANUMANTHAPPA KRISHNA PRASAD , (2) PRABHAKAR SAVITA, 3) MISHRA ANJALI, (4) SIVASWAMI TYAGI JAYA, of Department of Biotechnology, All India Institute of Medical Sciences, New Delhi-10029, India , all are Indian nationals.

4. I/We, claim the priority from the application(s) filed in convention countries, particulars of which are as follows :

NA

5. I/We state that the said invention is an improvement in or modification of the invention the particulars of which are as follows and of which I/We are the application/patentee:

6. I / We state that the application is divided out of my/our application, the particulars of which are given below and pray that this application be deemed to have been filed on NA under section 16 of the act.

7. That I am / We are the assignee of the true and first inventors.

8. That my / our address for service in India is as follows :
L S DAVAR & CO., of 5/1, 1st Floor, Kalkaji Extension, New Delhi-110 019 and
Monalisa, Flats IB & IC, 17, Camac Street,
Kolkata-700 017.
Phones : 247-3996, 247-5918, 280-5536
Fax No. : 91-33-247-5886, 240-6292
91-11-646-4443

9. Following declaration was given by the inventor(s) or applicant(s) in the convention country :

I / We the true and first inventors for this invention or the applicant(s) in the convention country declare that the applicant(s) herein is / are my / our assignee or legal representative.

Signature of the true and first Inventor/s or Applicant In the convention country with date, name to be given below Signature

HANUMANTHAPPA KRISHNA PRASAD

PRABHAKAR SAVITA

MISHRA ANJALI

SIVASWAMI TYAGI JAYA

10. That to the best of my / our knowledge, information and belief the fact and matters stated herein are correct and that there is no lawful ground of objection to the grant of patent to me / us on this application.

11. Following are the attachment with application :

(a) Provisional/~~Complete~~ specification (3 copies).

(b) Drawings 4 (Sheets) 3 copies. Informal

(c) Priority document/s NA

(d) Statement and undertaking on Form 3 in dupl.

(e) Form 5. NA

(f) Power of Authority. To Follow

(g)

(h)

(i) Fee Rs. ~~1,500/-~~ /Rs. 5,000/- in cheque / bank draft.

bearing No.....date.....

on.....Bank.

To be Signed by applicant or authorised patent agent

~~I~~/We request that a patent may be granted to ~~me~~/us for the said invention.

Dated this 16th day of December 2002

Signature (/. BANERJEE)

OF L-S-DAVAR & Co.
APPLICANTS' AGENT

To
The Controller of Patents
The Patent Office
at New Delhi

1274 DEL 02

FORM - 2

18 DEC 2002

THE PATENTS ACT, 1970

(39 of 1970)

PROVISIONAL/COMPLETE

SPECIFICATION

SECTION 10

ORIGINAL

TITLE

THE CHARACTERIZATION OF HUP B GENE ENCODING HISTONE LIKE PROTEIN OF MYCOBACTERIAL TUBERCULOSIS

APPLICANT

1) DEPARTMENT OF BIOTECHNOLOGY, of Block 2, 7th Floor, CGO Complex, Lodi Road, New Delhi-110 003;
2) ALL INDIA INSTITUTE OF MEDICAL SCIENCES, of Ansari Nagar, New Delhi-110 029.

The following specification particularly describes the nature of the invention and the manner in which it is to be performed

FIELD OF THE INVENTION

This invention relates to the characterization of hupB gene encoding histone like protein of mycobacterial tuberculosis

BACKGROUND OF THE INVENTION

Numerous techniques are in vogue to differentiate between members of the MTB complex. Several researchers have demonstrated that the use of IS6110 as a target for PCR amplification gives the best sensitivity and specificity in the diagnosis of tuberculosis. However when tested with standard mycobacterial species and strains obtained from ATCC and mycobacterial cultures isolated from clinical specimens, the target was limited in its ability to distinguish between M. tuberculosis complex from other mycobacteria.

Spoligotyping based on detection of nonrepetitive spacer sequences located between small repetitive units in the DR locus of the MTB complex strains, other genetic markers and biochemical tests have been used to differentiate between M. tuberculosis, M. bovis, M. africanum, M. microti, and M. canetti, (Niemann et al., 2000). Besides spoligotyping, mtp40 gene sequence (Liebana et al., 1996), pncA gene point mutation at position 169 (Scropio & Zhang, 1996), polymorphism of the oxyR locus (Sreevatsan et al., 1996), have been reported as useful targets for identification of the members of the TB complex. Ideally, the target for PCR based detection should be that it discriminates not only among mycobacterial species but also is able to distinguish between closely related members of the MTB complex.

Although PCR techniques have been widely evaluated in the diagnosis of tuberculosis, the reports have generally focused on the detection of M.

tuberculosis. However their success has been limited to differentiating between the tuberculosis complex and non-tuberculous mycobacteria. In the present study the hupB gene target has been shown to be a target which permits differentiation of M. tuberculosis from M. bovis and from among other members of the TB complex, non-tuberculous mycobacterial and non-mycobacterial species tested. The assay would prove useful especially in developing countries with a high incidence of infected livestock (Grange, 2001). M. bovis has been known to spread to humans from infected cattle by the aerosol route or by consumption of infected diary products (Zoonotic tuberculosis) (Moda et al., 1996; Cosivi et al., 1998). Although bovine tuberculosis has been largely eradicated in developed countries it continues to occur in developing countries. The epidemiological impact of bovine tuberculosis on human health has not been assessed and is a major lacuna in developing countries. However with reports of tuberculosis due to M. bovis in AIDS patients (Bouvet et al., 1993; O'Reilly et al., 1995) and with increasing incidence of tuberculosis globally, rapid and reliable diagnostic assays are required not only for detection but also identification of the pathogenic mycobacteria in clinical samples. This is essential for prompt diagnosis, treatment and control of tuberculosis. Here we report a PCR-RFLP assay that enables the precise identification of closely related mycobacteria belonging to the MTB complex.

hupB gene encoding histone-like protein of Mycobacterium tuberculosis has been exploited as a target for detection and differentiation of M. tuberculosis and M. bovis. Two methods were used to identify mycobacterial constituents associated with human response namely the T cell blot and immuno –

substraction assays (Prabhakar et al., 1998). The 30kDa fraction of the mycobacterial lysate was found to induce the highest lymphoproliferative index among the tuberculin reactors. In immuno-subtraction assays a prominent reactive band was similarly seen at approximately 30 kDa. The30kDa protein was electroeluted from the SDS-PAGE gel and purified to homogeneity.

Using the internal peptide sequence (VKPTSVPAFRPGAQFK) a 100% identity was obtained with 16 amino acids of the cosmid CY349. The corresponding gene was later annotated and designated as the hupB gene (Rv 2986c, Cole et al., 1998). The protein was found to be localized in the cytoplasm and on the cytoplasmic surface of the mycobacterial membrane. The hupB gene has been classified among the DNA binding (histone like) proteins of M. tuberculosis (Cole et al., 1998). Primers were designed to amplify the hupB gene. A 645 bp amplicon was obtained in case of M. tuberculosis. The

$\alpha^{32}P$ labelled PCR amplicon was used in Southern hybridization to establish the size, prevalence and organisation of the HupB gene in members of the MTB complex (M. tuberculosis and M. bovis) and other mycobacterial species.

OBJECTS OF THE INVENTION

An object of this invention is to characterize hupB gene encoding histone like protein of mycobacterial tuberculosis.

Another object of this invention is to characterize hupB gene which can be utilized to distinguish and identify the mycobacterial species belonging to the MTB complex.

Further object of this invention is to characterize mycobacterial gene as new targets for novel anti-mycobacterial chemotheraputic agents.

BRIEF DESCRIPTION OF THE INVENTION

According to this invention the size variability of the hupB gene was determined. A set of primers were designed to amplify the C-terminal part of the hupB gene. Mycobacterial DNA extracted from M. tuberculosis and M. bovis were used. PCR amplified product was obtained in both M. tuberculosis and M. bovis. However the amplicon obtained in case of M. bovis was slightly smaller than that obtained in case of M. tuberculosis. This difference was confirmed by analyzing over 50 M. tuberculosis and M. bovis strains collected from diverse sources (Table1). The DNA extracted from 3 standard strains and 4 clinical isolates of M. tuberculosis and M.bovis (BCG) were included for amplification using the hupB primers (HLPmtNI and HLPmtSI, Table II). The difference in the size of amplicons obtained in case of M. tuberculosis and M. bovis was validated by RFLP and confirmed by sequencing of the PCR products. The PCR products of the two mycobacteria were digested with HaeIII and HpaII and analysed on 12% non-denaturing gel. Digestion of the 645 bp product with HpaII revealed that a ~ 250 bp fragment was seen in case of M. bovis compared to the band of ~ 280 bp size obtained in case of M. tuberculosis, (Fig.3). Analysing the sequence of the PCR products showed that in M. bovis there was a deletion of 27 bp corresponding to 9 amino acids, (Fig.2). As a result of this deletion

the PCR amplicon obtained in case of M. bovis was 618 bp, 27 bp smaller than the PCR product obtained in case of M. tuberculosis (645 bp).

In the present study the hupB gene target has been shown to be one such target which permits differentiation of M. tuberculosis from M. bovis and from among other members of the TB complex, mycobacterial and non-mycobacterial species tested. The assay would prove useful especially in developing countries with a high incidence of infected livestock (Grange, 2001). M. bovis has been known to spread to humans from infected cattle by aerosol or by consumption of infected diary products (Zoonotic tuberculosis) (Moda et al., 1996; Cosivi et al., 1998). Although bovine tuberculosis has been largely eradicated in developed countries it continues to occur in developing countries. The epidemiological impact of bovine tuberculosis on human health has not been assessed and is a major lacuna in developing countries.

Fig. : 1 Specificity analysis of $hupB_{Mt}$ bsed PCR assay

Amplification products were electrophoresed on agarose gels. Their ethidium bromide staining (Panel A & Panel B) and hybridization profiles have been shown in Panel A' & Panel B' respectively. The 645 bp product has been indicated. Panels A & A'; Lanes 1 M. tuberculosis H37 Rv; 2, M. tuberculosis H37Ra; 3, M.bovis BCG; 4, M. microti; 5, M. xenopi; 6, M.fortuitum; 7, M. phlei; 8, M.gordonae; 9, M.vaccae; 10, M. kansasii; 11, 100 bp Marker; 12, M. intracellulare; 13, M. avium; 14, M. scrofulaceum; 15, M. smegmatis; 16, M. tuberculosis P8497; 17, M. tuberculosis C1084; 18, M. tuberculosis 779634; 19, M. chelonei; 20, M. tuberculosis P8473; 21, M. gastri.

Panel B & B' Lanes 1, M. tuberculosis 1207; 2, E. coli; 3, N.asteroids; 4, S. aureus; 5, P. aeruginosa; 6, S. faecalis; 7, S. aureus; 8, A. niger; 9, A. fumigatus; 10, C. albicans; 11, 100 bp marker; 12, M. tuberculosis Erdman; 13, K. pneumoniae; 14, M. leprae; 15, M. africanum; 16, Negative control. Hybridisation in panels B & B' was carried out with 645 bp fragment (PstI & NcoI digest from the plasmid pHLPMT).

Fig.2: Sensitivity of detection of M. tuberculosis DNA by hupB based PCR assay.

Amplification reactions were performed with serial dilutions of M. tuberculosis DNA (1ng to 1fg). The ethidium bromide and hybridization patterns are seen in panels A and B respectively. The 645 bp product has been indicated. Lanes 1, 1ng; 2, 500pg; 3, 50 pg; 4, 5pg; 5, 1pg; 6, 500fg; 7,100fg; 8,50fg; 9,10fg; 10,5fg; 11, 2fg; 12, 1fg; 13, Negative control; 14, positive control (M. tuberculosis); M, λ DNA HindIII digest. The detection limit was 50 pg by ethidium bromide staining and 500 fg for hybridization.

Fig.3: RFLP analysis of the 645 and 318bp PCR products.

Panel A depicts the schematic representation of the position of the primers in the hupB sequence, which were used in order to obtain the 645 bp and 318 bp PCR products. Ethidium bromide staining for 645 bp (Panel B) and 318 bp (Panel C) amplification products are shown. Lanes 1, M. tuberculosis H37Rv; 2, M. tuberculosis H37Ra; 3, M. tuberculosis Erdman; 4, M. bovis AN5; 5, M. bovis BCG (Japan); 6, M. bovis BCG (Copenhagen); 7, M. bovis IC 378; 8, M. bovis IC 379; 9, M. bovis IC 380; 10, M. bovis IC 381; 11, M. bovis IC 382; 12, PCR molecular weight marker. Panel D, RFLP

poly-acrylamide gel analysis of 645 bp amplicon digested with Hpall (lanes 1-3) and HaeIII (lanes 6-9): Lane 1, M. tuberculosis H37Rv; 2, M. tuberculosis H37Ra; 3, M. bovis BCG; 4, Negative control; 5, 100 bp Molecular weight marker; 6, M. tuberculosis H37Rv; 7, M. tuberculosis H37Ra; 8, M. bovis BCG; 9, M bovis AN5.

Fig.4: Nucleotide sequence alignment of hupB gene of M.tuberculosis and M.bovis

The nucleotide sequence of the C-terminal region (326-676 bp) of hupB gene of standard strains of M. tuberculosis and M. bovis and clinical isolates of M. bovis has been aligned using GCG software. A deletion of 27 bp was seen in hupB sequence of all M. bovis strains. The 9 deleted amino acids (KAATKAPAR) between 385 to 411bp with respect to M.tuberculosis are shown in single letter code on the first line. Numbers in brackets refer to nucleotide position in hupB (Rv2986c). The M.bovis strain numbers are given on the left.

Table 1: Mycobacterial and Non-mycobacterial Species and Strains used in the PCR Assay

Species	Strain No.
M.tuberculosis (Human isolates)	H37Rv, H37Ra, Erdman, P8473, P8497, C1207, C1084,779634, ICC107, ICC120, ICC22, ICC238, ICC136, ICC37, ICC247, ICC16, ICC235, ICC145, ICC06, ICC11, ICC85, ICC95, CSU-17, CSU-27, CSU-20
M.bovis (Cattle Isolates)	T11, AN5, IC378, IC379, IC380, IC381, IC382, ICC388, ICC391, 117, 126, 73, 130 CL1, CL3, CL4, CL8, CL10, CL33, CL42, Japanese* & Copenhagen*
Human Isolates	6, 47, 85,
M. canetti	116
M. africanum	81543
M. microti	OV254, T14, N5
M. gastri	TMC1456
M. chelonae	TMC191, J31
M. vaccae	IND123
M. avium	NCTC8562, ICC192
M. intracellulare	TMC1302, N25, N8
M. scrofulaceum	TMC1302, MAC29
M. gordonae	TMC1324
M. fortuitum	SJ32, ICC420, ICC419, ICC417, ICC416
M. smegmatis	ATCC27204, LR222, N18
M. phlei	ND124, N14
M. Kansasii	1201
M. leprae	Tissue Biopsy
M. simae	IN7
Corynebacterium diphtheriae	Clinical isolate
Streptococcus β-haemolyticus	Clinical isolate
Staphylococcus aureus	Clinical isolate
Pseudomonas aeruginosa	Clinical isolate
Klebsiella pneumoniae	Clinical isolate
Nocardia asteriodes	MTCC274
Aspergillus fumigatus	Soil isolate
Aspergillus niger	Soil isolate
Candida albicans	Clinical isolate
Escherichia coli	DH5$_\alpha$, BL21 (DE3)

Table II

Primers Used for Amplification of hupB Mycobacterial DNA Target
1) HLPmtNI (5' ggagggttgggatgaacaaagcag 3')
2) HLPmtSI (5' gtatccgtgtgtcttgacctatttg 3')
(The expected size of the amplicon was ~645 bp.)

Dated this 16 th day of DECEMBER 2002.

of L S DAVAR & CO.,
Applicants' Agent

FORM-2

THE PATENTS ACT, 1970

(39 of 1970)

~~PROVISIONAL~~/COMPLETE

SPECIFICATION

SECTION 10

TITLE

THE CHARACTERIZATION OF HUP B GENE ENCODING HISTONE LIKE PROTEIN OF MYCOBACTERIAL TUBERCULOSIS

APPLICANT

1) DEPARTMENT OF BIOTECHNOLOGY, of Block 2, 7th Floor, CGO Complex, Lodi Road, New Delhi 110 003; and
2) ALL INDIA INSTITUTE OF MEDICAL SCIENCES, of Ansari Nagar, New Delhi-110 029.

The following specification particularly describes the nature of the invention and the manner in which it is to be performed

FIELD OF THE INVENTION

This invention relates to the characterization of *hupB* gene encoding histone like protein of *Mycobacterium tuberculosis*.

BACKGROUND OF THE INVENTION

Numerous techniques are in vogue to differentiate between members of the MTB complex. Several researchers have demonstrated that the use of *IS6110* as a target for PCR amplification gives the best sensitivity and specificity in the diagnosis of tuberculosis. However when tested with standard mycobacterial species and strains obtained from ATCC and mycobacterial cultures isolated from clinical specimens, the target was limited in its ability to distinguish between *M. tuberculosis* complex from other mycobacteria. Spoligotyping based detection of non-repetitive spacer sequences located between small repetitive units in the DR locus of the MTB complex strains, other genetic markers and biochemical tests have been used to differentiate between *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, and *M. canetti*, (Niemann et al., 2000). Besides spoligotyping, *mtp40* gene sequence (Liebana et al., 1996), *pncA* gene point mutation at position 169 (Scropio & Zhang, 1996), polymorphism of the *oxyR* locus (Sreevatsan et al., 1996), have been reported as useful targets for identification of the members of the TB complex.

Ideally, the target for PCR based detection should be that it discriminates not only among mycobacterial species but also is able to distinguish between closely related members of the MTB complex.

Although PCR techniques have been widely evaluated in the diagnosis of tuberculosis, the reports have generally focused on the detection of *M.*

tuberculosis. However their success has been limited to differentiating between the tuberculosis complex and non-turberculous mycobacteria.

Here we report a PCR assay that enables the precise identification of closely related mycobacteria belonging to the MTB complex. *hupB* gene encoding histone-like protein of *M. tuberculosis* has been exploited as a target for detection and differentiation of *M. tuberculosis* and *M. bovis*. The *hupB* gene target not only permits differentiation of *M. tuberculosis* from *M. bovis*, but also from among other members of the MTB complex, non-tuberculous mycobacteria as well as non-mycobacterial species tested. The assay would prove useful especially in developing countries with a high incidence of infected livestock (Grange, 2001). *M. bovis* has been known to spread to humans from infected cattle by the aerosol route or by consumption of infected diary products (Zoonotic tuberculosis) (Moda et al., 1996; Cosivi et al., 1998). Although bovine tuberculosis has been largely eradicated in developed countries it continues to occur in developing countries. The epidemiological impact of bovine tuberculosis on human health has not been assessed and is a major lacuna in developing countries. However with reports of tuberculosis due to *M. bovis* in AIDS patients (Bouvet et al., 1993; O'Reilly et al., 1995) and with increasing incidence of tuberculosis globally, rapid and reliable diagnostic assays are required not only for detection but also identification of the pathogenic mycobacteria in clinical samples. This is essential for prompt diagnosis, treatment and control of tuberculosis.

Immunogenecity of HupB protein: Two methods were used to identify mycobacterial constituents associated with human response namely the T cell blot and immuno – subtraction assays (Prabhakar et al., 1998). The 30kDa fraction of the mycobacterial lysate was found to induce the highest lymphoproliferative index among the tuberculin reactors. In immuno-subtractive assays a prominent reactive band was similarly seen at approximately 30 kDa. The30kDa protein was electro-eluted from the SDS-PAGE gel and purified to homogeneity.

Using the internal peptide sequence (VKPTSVPAFRPGAQFK) a 100% identity was obtained with 16 amino acids of the cosmid CY349. The corresponding gene was later annotated and designated as the *hupB* gene (Rv 2986c, Cole et al., 1998). The protein was found to be localized in the cytoplasm and on the cytoplasmic surface of the mycobacterial membrane, by immuno-gold electron microscopy. The *hupB* gene has been classified among the DNA binding (histone like) proteins of *M. tuberculosis* (Cole et al., 1998). Primers were designed to amplify the *hupB* gene. A 645 bp amplicon was obtained in case of *M. tuberculosis*. The $\alpha^{32}P$ labeled PCR amplicon was used in Southern hybridization to establish the size, prevalence and organization of the *hupB* gene in members of the MTB complex (*M. tuberculosis* and *M. bovis*) and other mycobacterial species.

OBJECTS OF THE INVENTION

An object of this invention is to characterize *hupB* gene encoding histone like protein of *M. tuberculosis*.

The another object of this invention is to characterize *hupB* gene which can be utilized to distinguish and identify the mycobacterial species belonging to the MTB complex.

Further object of this invention is to characterize mycobacterial gene as new targets for novel anti-mycobacterial chemotheraputic agents.

SUMMARY OF THE INVENTION

According to this invention there is provided means for identification of *hupB* gene encoding histone like protein a target for detection *M. tuberculosis* and *M. bovis*.
Further there is provided a process for differentiating of the *hupB* gene of *M.tuberculosis* and *M. bovis*.

DESCRIPTION OF THE INVENTION

The size variability of the *hupB* gene was determined using 3 sets of primers (Fig: 1, Table II) :

1) ***hupB* gene target DNA** Primers N (5' ggagggttgggatgaacaaagcag 3') and S (5' gtatccgtgtgtcttgacctatttg 3') were used to amplify *hupB* gene sequences. The expected size of the amplicon was 645bp in case of *M. tuberculosis*, and 618 bp in case of *M. bovis* respectively.

2) **The C- terminal portion of the gene**: was also amplified by using :

(i) internal primer M (5' gcagccaagaaggtagcgaa 3') with S (5' gtatccgtgtgtcttgacctatttg 3'), the expected amplicon was ~ 318 bp, (Fig:1).

The expected size of the amplicon was 318 bp in case of *M. tuberculosis*, and 291 bp in case of *M. bovis* respectively.

(ii) using primers F (5' ccaagaaggcgacaaagg3') with R (5' gacagctttcttggcgggg3'). The expected size of the amplicon was 116 bp in case of *M. tuberculosis*, and 89 bp in case of *M. bovis* respectively.

Mycobacterial DNA extracted from *M. tuberculosis* and *M. bovis* were used. PCR amplified product was obtained in both *M. tuberculosis* and *M. bovis*. However the amplicon obtained in case of *M. bovis* was slightly smaller than that obtained in case of *M. tuberculosis*. This difference was confirmed by analyzing over 50 *M. tuberculosis* and *M. bovis* strains collected from diverse sources (Table I). The DNA extracted from 3 standard strains and 4 clinical isolates of *M. tuberculosis* and *M. bovis* (BCG) were included for amplification using the *hupB* primers (N and S, / M and S, Table II). The difference in the size of amplicons obtained in case of *M. tuberculosis* and *M. bovis* was validated by RFLP (Fig: 4D) and confirmed by sequencing of the PCR products, (Fig: 5). The PCR products of the two mycobacteria were digested with *HaeIII* and *HpaII* and analyzed on 12% non-denaturing gel. Digestion of the 645 bp product with *HpaII* revealed that a ~ 250 bp fragment was seen in case of *M. bovis* compared to the band of ~ 280 bp size obtained in case of *M. tuberculosis*, (Fig: 4D). Analyzing the sequence of the PCR products showed that in *M. bovis* there

was a deletion of 27 bp corresponding to 9 amino acids, (Fig: 5). As a result of this deletion the PCR amplicon obtained in case of *M. bovis* was 618 bp, 27 bp smaller than the PCR product obtained in case of *M. tuberculosis* (645 bp), (Fig: 4 B,C).

Results, obtained with the amplicon generated in the C - terminal portion of the gene using **M** and **S** primers on digestion with *Hpa*II, showed differences matching to the differences seen in case of the PCR product obtained using the *hupB* primers (**N** and **S**) indicating that the PCR-RFLP assay utilizing either the PCR product obtained using the *hupB* primers (**N** and **S**) / the C terminal primers (**M** and **S**) did distinguish between *M. tuberculosis* and *M. bovis*.

The utility of the *hupB* gene as a target in diagnosis and identification pathogenic mycobacteria in bovine tuberculosis has been demonstrated, (Table IV-VII). The sensitivity and specificity of the assay showed remarkable improvement with the adoption of the **nested PCR technique** in clinical samples, targeting the C-terminal part of the *hupB* gene, (Fig: 6) and (Table VI- VIII).

EXAMPLES

Bacterial strains: The mycobacterial strains as well as non-mycobacterial strains used in the study have been listed in Table I. In all 80 mycobacterial strains were included in the study besides 10 non-mycobacterial species. Of the 80 mycobacterial isolates included 55 were members of the MTB complex, (*M. tuberculosis* – 25, *M. bovis* – 25, *M. microti*-3, and 1 each of *M. africanum* and *M. canetti*). The details of the *M. bovis* strains included are as follows: 7 from infected cattle housed in the Central Military Veterinary Laboratory, Meerut, India, 9 from National Mycobacterial Repository, JALMA, Agra India, 2 each from Netherlands and Argentina and 3 human isolates from the Netherlands (Drs.J.D.A. van Embden and D.van Soolingen).

Processing of bacilli for specificity analysis

All the mycobacterial and non-mycobacterial strains grown on solid media (LJ slants all mycobacterial species), LB agar (*E. coli*) nutrient agar (*Aspergilus niger*, *Nocardia asteriodes*, *Pseudomonas aeruoginosa*, *Klebsiella pneumoniae*) or blood agar (*Corynebacterium diphtheriae*, *Streptococcus pneumoniae*) were scraped with the help of sterile toothpicks and re-suspended in sterile distilled water containing 0.1% Triton X-100. Re-suspended bacilli were boiled at 100°C for 20 minutes and an aliquot (2µl) was used for PCR.

PCR Analysis:

1) 23S rDNA target: Primers: C*(5' gtgagcgacgggatttgcctat 3') and L*(5' accacccaaaaccggatcgat 3') were used to detect the presence of DNA from organisms belonging to genus Mycobacterium. The expected size of the amplicon was 174bp (Verma et al., 1994; Dasgupta et al., 1998).

2) *hupB* DNA target : Primers N (5' ggagggttgggatgaacaaagcag 3') and S (5' gtatccgtgtgtcttgacctatttg 3') were used to amplify *hupB* gene sequences. The expected size of the amplicon was ~645 bp (Table II, Fig:1).

Each reaction (20µl) contained 1.5 mM $MgCl_2$, 0.5 µM of primers; 200 µM dNTPs, 10 mM Tris-HCl (pH 8.8 at 25°C), 50 mM KCl, 0.08% Nonidet P40, and 0.5 Units of Taq DNA Polymerase. The PCR reaction was subjected to initial denaturation at 94°C for 10 min., and 35 cycles of each of 1 min at 94°C, 1 min., at 63°C and 1 min at 72°C followed by final extension at 72°C for 30 mins. The products were analyzed on a 1.2 % agarose gel and stained with ethidium bromide.

The C-terminal portion of the gene was amplified by using M (5' gcagccaagaaggtagcgaa 3') with S (5' gtatccgtgtgtcttgacctatttg 3'), the expected amplicon was ~ 318 bp.

Nested PCR: The target DNA used in nested PCR was the PCR product obtained using the primers N and S. The target area of the C terminal part of

the *hupB* gene was amplified by using F (5' ccaagaaggcgacaaagg3') with R (5' gacagctttcttggcgggg3'), the expected amplicon was ~ 116 bp in case of *M. tuberculosis* and 89 bp in case of *M. bovis*, (Table II, Fig:1).

Each reaction (40µl) contained 2.5 mM $MgCl_2$, 0.5 µM of primers, 200 µM dNTPs, 10 mM Tris-HCl (pH 8.8 at 25°C), 50 mM KCl, 0.08% Nonidet P40, and 0.5 Units of Taq DNA Polymerase. The PCR reaction was subjected to initial denaturation at 95°C for 10 min., and 35 cycles of 1 min at 94°C, 1 min., and 30 seconds at 59°C and final extension at 72°C for 7 mins. The products were analyzed on a 3.5 % agarose gel / 8 % non-reducing polyacrylamide gel and stained with ethidium bromide.

Southern Hybridization: The PCR amplicons resolved on the agarose gel were transferred on to nitro-cellulose membrane (Southern, 1975). The blots were then hybridized with α-^{32}P labeled 645 bp *hupB* gene probe from *M. tuberculosis*.

Restriction Fragment Length Polymorphism:

hupB amplified sequences were digested with HpaII and HaeIII restriction enzyme and the products were analyzed on a 12% non-denaturing polyacrlyamide gel. The gel was strained with ethidium bromide and DNA fragments were visualized under ultraviolet light.

DNA Sequencing Analysis: The PCR products were sequenced by the Sanger's dideoxy chain termination method (Sanger et al., 1977) using Sequenase Ver 2.0 sequencing kit, α^{35} SdATP and forward/reverse universal M13 primers or internal primers of *hupB*, according to the manufacturer's instructions. The DNA template was alkali denatured and annealed to the primers at -70°C for 1 hour. The GC rich mycobacterial DNA was mixed with 0.5 µg of single strand binding protein prior to labeling. The protein was digested with proteinase K 0.1 µg at 68°C for 20 mins., following termination of the labeling reaction. The reactions were electrophoresed on a 6% urea –polyacrylamide gel in 1X TBE at 70 W for a suitable time period. The gel was fixed with acetic acid (10%) and methanol (30%) dried and autoradiographed. The PCR products obtained in standard strains and isolates were also sequenced commercially by Microsynth, Switzerland.

The specificity of the PCR assay: DNA from 16 mycobacterial and 10 non-mycobacterial species were used as target to establish the specificity of the PCR assay, (Table 1). The DNA extracted from 3 standard strains and 4 clinical isolates of *M. tuberculosis* and *M. bovis* (BCG) were included for amplification using the *hupB* primers (N and S, Table II, Fig: 1). Only in case of *M. tuberculosis* H37Rv, H37Ra, *M. bovis* BCG and 5 clinical isolates of *M. tuberculosis* (lanes:1, 2,3,16,17,18 and 20, in Fig:2A and lanes 1 and 12 in Fig.2B) the expected 645 bp product was obtained. No amplification was seen with *M. microti*, *M. africanum* of the MTB complex, *M. leprae*, MAIS complex and other mycobacterial species (rapid and slow growers) including *Corynebacterium diphtheriae* and *Nocardia asteriodes* that together make the CNM group. Amplification was also not seen in other non-mycobacterial species (Fig:2B). The authenticity of the amplified

product was confirmed by hybridization with α-^{32}P labeled 645 bp fragment (released by *PstI* and *NcoI* digestion from plasmid pHLPMT containing *hupB* gene of *M. tuberculosis*) (Fig: 2A' and B'). This confirmed that no other amplification was obtained with any other template DNA that could have been missed by ethidium bromide staining alone. Thus the 5' and 3' primers of *hupB* are specific for *M. tuberculosis* and *M. bovis*.

Sensitivity of *hupB* gene based PCR assay: The sensitivity of DNA PCR amplification (level of detection) was established by adding serial dilutions of mycobacterial DNA (1 ng to 1 fg) in the PCR reaction using primers N and S. It was seen that by ethidium bromide staining alone the detection limit was 50 pg and by hybridization the detection limit increased to 500 fg (Fig: 3A and B). This was equivalent to the detection of 5000 and 50 genome equivalents respectively.

RFLP of PCR Amplicons of *hupB* gene derived from *M. tuberculosis* and *M. bovis*: DNA from different isolates of *M. tuberculosis* and *M. bovis* (listed in Table I) were amplified using (i) N and S primers (645 bp product, Table II) and (ii) M (internal primer) and S (318 bp product, Fig: 4C, Table II, Fig: 1). PCR amplicons obtained from the DNA of *M. bovis* strains (lanes 4-11, Fig: 4B and 4C) were smaller in size as compared to the PCR amplicons obtained from the *M. tuberculosis* strains (lanes 1-3, Fig:4B and 4C). The results of the PCR assay with the 2 sets of primers have been summarized in Table III. The 645 and 318 bp amplicons were obtained in all tested strains except in case of 4 *M. bovis* isolates obtained from the Netherlands. In these isolates 645 bp product was not obtained however the 318 bp was detected.

In order to confirm the difference in PCR product sizes, the amplicons were digested with *Hpall* and *Haelll* (Fig.:4D). The digested products were analysed on 12% non-denaturation polyacrylamide gel. Digestion of 645 bp product with *Hpall* clearly revealed that in case of *M. bovis* a ~250 bp (Fig: 4D, lane 3) product obtained was smaller in size compared to the ~280 bp bands obtained with *M. tuberculosis* H37Ra & H37Rv (Fig: 4D, lanes 1 and 2). No differences were perceived with *Haelll* digestion, (Fig: 4D, lanes 5-8). Results, obtained with the amplicon (318 bp) generated in the C – terminal portion of the gene using **M** and **S** primers on digestion with *Hpall*, showed similar differences (results not shown) indicating that the PCR-RFLP assay did distinguish between *M. tuberculosis* and *M. bovis* strains.

Sequencing of PCR Amplified Product: PCR amplicons obtained from DNA of standard strains of *M. bovis* and *M. tuberculosis* including local isolates of *M. bovis* derived from cattle were sequenced. Sequence analysis indicated that in *M. bovis* there was a deletion of 27 bp (9 amino acids) in frame after 128th codon in the C terminal part of the gene (Fig: 5). The histone like gene sequence of *M. bovis* has been submitted to the NCBI data base (Accession No.Y18421).

FIGURE LEGENDS:

Fig: 1 Position of the *hupB* gene and Primers used to generate PCR products.

Panel A: The position of the primers in the *hupB* sequence, which were used in order to obtain the PCR products have been depicted. Primer pairs N & S specific for the *hupB* gene; Internal primer M & S specific for the C terminal part of the *hupB* gene .

Panel B, C and D : The ethidium bromide stained amplification products of *M. tuberculosis* and *M. bovis* generated using primer pairs N & S (Panel B), M & S (Panel C) and F & R (Panel D) were electrophoresed on polyacrylamide gels. The 645 and 618 bp (Panel B); 318 and 291 bp (Panel C); 116 and 89 bp (Panel D); products have been indicated . Lanes 1 & 4, 645 bp, 6 & 10, 318 bp , and 13, 116 bp of the of *hupB* gene / C terminal part of the gene amplification product obtained in *M. tuberculosis* H37Rv ; lanes 2 & 5, 618 bp of *hupB* gene, 7 & 9, 291 bp and 11,12,15-17, 89 bp of the *hupB* gene / C terminal part of the gene amplification product obtained in *M bovis* AN5 ; 3, 8 & 14, 100 bp molecular weight markers.

Fig: 2 Specificity analysis of $hupB_{Mt}$ based PCR assay

Amplification products were electrophoresed on agarose gels. Their ethidium bromide staining (Panel A & Panel B) and hybridization profiles have been shown in Panel A' & Panel B' respectively. The 645 bp product has been indicated. Panels A & A'; Lanes 1 *M. tuberculosis* H37 Rv ; 2, *M. tuberculosis* H37Ra ; 3, *M. bovis* BCG ; 4, *M. microti* ; 5, *M. xenopi* ; 6, *M. fortuitum* ; 7, *M. phlei* ; 8, *M. gordonae* ; 9, *M. vaccae* ; 10, *M. kansasii* ; 11, 100 bp Marker ; 12 , *M. intracellulare* ; 13, *M. avium* ; 14, *M.*

scrofulaceum ; 15, *M. smegmatis* ; 16, *M. tuberculosis* P8497 ; 17, *M. tuberculosis* C1084 ; 18, *M. tuberculosis* 779634 ; 19, *M. chelonei* ; 20, *M. tuberculosis* P8473 ; 21, *M. gastri.*

Panel B & B' Lanes 1, *M. tuberculosis* 1207; 2, *E. coli*; 3, *N. asteroides* ; 4, *S. aureus* ; 5, *P. aeruginosa* ; 6, *S. faecalis* ; 7, *S. aureus* ; 8, *A. niger*; 9, *A. fumigatus* ; 10, *C. albicans* ; 11, 100 bp marker ; 12, *M. tuberculosis* Erdman ; 13, *K. pneumoniae* ; 14, *M. leprae* ; 15, *M. africanum* ; 16, Negative control. Hybridisation in panels B & B' was carried out with 645 bp fragment (*Psti* & *Nco*I digest from the plasmid pHLPMT) .

Fig: 3 **Sensitivity of detection of *M. tuberculosis* DNA by *hupB* based PCR assay.**

Amplification reactions were performed with serial dilutions of *M. tuberculosis* DNA (1ng to 1 fg). The ethidium bromide and hybridisation patterns are seen in panels A and B respectively. The 645 bp product has been indicated. Lanes 1, 1 ng ; 2, 500pg ; 3, 50 pg ; 4, 5pg ; 5, 1 pg ; 6, 500 fg ; 7, 100 fg ; 8, 50 fg ; 9, 10 fg ; 10, 5 fg ; 11, 2 fg ; 12, 1 fg ; 13, Negative control ; 14, positive control (*M. tuberculosis*) ; M, λ DNA *Hind*III digest. The detection limit was 50 pg by ethidium bromide staining and 500 fg for hybridisation.

Fig: 4 **RFLP analysis of the 645 and 318bp PCR products.**

Panel A depicts the schematic representation of the position of the primers in the *hupB* sequence, which were used in order to obtain the 645 bp and 318 bp PCR products. Ethidium bromide staining for 645 bp (Panel B) and 318 bp (Panel C) amplification products are shown. Lanes 1, *M. tuberculosis* H37Rv ; 2, *M. tuberculosis* H37Ra ; 3, *M. tuberculosis* Erdman ; 4, *M. bovis* AN5 ; 5, *M. bovis* BCG (Japan) ; 6, *M. bovis* BCG (Copenhagen) ; 7, *M. bovis* IC 378 ; 8, *M. bovis* IC 379; 9, *M. bovis* IC 380 ; 10, *M. bovis* IC 381 ; 11, *M. bovis* IC 382 ; 12, PCR molecular weight marker. Panel D, RFLP poly- acrylamide gel analysis of 645 bp amplicon

digested with *Hpa*II (lanes 1- 3) and *Hae*III (lanes 6-9) : Lane 1, *M. tuberculosis* H37Rv ; 2, *M. tuberculosis* H37Ra ; 3, *M. bovis* BCG ; 4, Negative control; 5, 100 bp Molecular weight marker ; 6, *M. tuberculosis* H37Rv; 7, *M. tuberculosis* H37Ra; 8, *M bovis* BCG; 9, *M bovis* AN5.

Fig: 5 Nucleotide sequence alignment of *hupB* gene of *M. tuberculosis* and *M. bovis*:

The nucleotide sequence of the C-terminal region (326-676 bp) of *hupB* gene of standard strains of *M. tuberculosis* and *M. bovis* and clinical isolates of *M. bovis* has been aligned using GCG software. A deletion of 27 bp was seen in *hupB* sequence of all *M. bovis* strains. The 9 deleted amino acids (KAATKAPAR) between 385 to 411bp with respect to *M. tuberculosis* are shown in single letter code on the first line. Numbers in brackets refer to nucleotide position in *hupB* (*Rv2986c*). The *M.bovis* strain numbers are given on the left.

Fig: 6 **Nested PCR Profile of *M. tuberculosis* and *M. bovis* Standard and Cattle derived Isolates :**

The nested PCR amplified products of the mycobacterial strains were electrophoresed on native 8% polyacrylamide gel, shown in Lanes 1 negative control; 2 molecular markers; 3 *M. tuberculosis* (H37Rv); 3 Cattle isolate identified as *M. tuberculosis*; 4 Cattle isolate identified as *M. bovis*; *M. bovis* (ICC380); and 5 *M. tuberculosis* (JALMA, Agra, Isolate).

Table I: Mycobacterial and Non-mycobacterial Species and Strains used in the PCR Assay

Species	Strain No.	Source
M.tuberculosis (Human isolates)	H37Rv, H37Ra, Erdman, P8473, P8497, C1207, C1084, 779634, ICC107, ICC120, ICC22, ICC238, ICC136, ICC37, ICC247, ICC16, ICC235, ICC145, ICC06, ICC11, ICC85, ICC95, CSU-17, CSU-27, CSU-20	a,b,c,d,,g n
M. bovis (Cattle Isolates)	T11, AN5, IC378, IC379 , IC380, IC381, IC382, ICC388, ICC391, 117, 126, 73, 130 , CL1, CL3 , CL4 , CL8 , CL10 , CL33 , CL42 , Japanese* & Copenhagen*	d,o,p,q
Human Isolates	6 ,47 , 85 ,	o
M. canetti	116	o
M. africanum	81543	e,g
M. microti	OV254, T14,N5	d,f
M. gastri	TMC1456	b
M. chelonae	TMC191,J31	b
M. vaccae	IND123	b
M. avium	NCTC8562, ICC192	d
M intracellulare	TMC1302, N25, N8	d
M. scrofulaceum	TMC1302, MAC29	d
M. gordonae	TMC1324	d
M. fortuitum	5J32, ICC420, ICC419, ICC417, ICC416	g,d,l
M. smegmatis	ATCC27204, LR222, N18	b,d
M. phlei	ND124, N14	b
M. Kansasii	1201	c
M. lepraе	Tissue Biopsy	d
M. simae	IN7	d
Corynebacterium diphtheriae	Clinical isolate	h
Streptococcus β- haemolyticus	Clinical isolate	h
Staphylococcus aureus	Clinical isolate	h
Pseudomonas aeruginosa	Clinical isolate	h
Klebsiella pneumoniae	Clinical isolate	h
Nocardia asteriodes	MTCC274	i
Aspergillus fumigatus	Soil isolate	j
Aspergillus niger	Soil isolate	j
Candida albicans	Clinical isolate	k
Escherichia coli	DH5α, BL21 (DE3)	m

a- P.S. Murthy, UCMS, University of Delhi, India; b- N.K. Jain, NDTC, New Delhi, India; c- C.N. Paramasivan, TRC, Chennai, India; d- V.M. Katoch, JALMA, Agra, India; e- Y.M. Yates, Public Health Laboratory, Dulwich Hospital, London, UK; f- P. Draper, NIMR, Mill Hill, London, UK; g- Kathleen Eisenach, University of Arkansas, USA; h- Dept. of Microbiology,AIIMS, New Delhi, India; i- Microbiological Type Culture Collection, IMTECH, Chandigarh, India; j- Shivkumar, Anna University, Chennai, India; k- Z.U. Khan, V.P. Chest Institute, Delhi, India; l- Jack Crawfort, CDC, Atlanta, GA, USA; m-GIBCO BRL, USA; n-Suman Laal, VA Medical Center, NY U, School of Medicine, New York, USA; o- J.D.A. van Embden, Netherlands; p-Central Military Veterinary Laboratory , Meerut, India; q- Dept. of Paediatrics, AIIMS, New Delhi, India; (*) Human vaccine strain; Numbers in bold - human isolates.

able II: Primers Used for Amplification of *hupB* Mycobacterial DNA Target

mer air	Sequence of Primer	Target *hup B* gene	Mycobacteria	PCR Product Size
N S	(5'ggagggttgggatgaacaaagcag 3') (5' gtatccgtgtgtcttgacctatttg 3')	Whole gene	*M. tuberculosis*	645 bp
			M. bovis	618 bp
I S	(5' gcagccaagaaggtagcgaa 3') (5' gtatccgtgtgtcttgacctatttg 3'),	C terminal	*M. tuberculosis*	318 bp
			M. bovis	291 bp
: t	(5' ccaagaaggcgacaaagg3') (5' gacagctttcttggcggg3').	C terminal	*M. tuberculosis*	116 bp
			M. bovis	89 bp

Table III: Representative results of *hupB* PCR Assay with Strains of *M.tuberculosis* and *M. Bovis*

Species	Strain	Source	645 / 318 bp
M.tuberculosis	H37Rv	ATCC[a]	+ / +
	H37Ra	" "	
	Erdman	" "	
		Human isolate [b]	
	779634	" "	+ / +
	P8473	" "	
	P8497	" "	
	C1207	" "	
	C1084		
M. bovis	AN5	Cattle isolate [c]	+ / +
	IC378	" "	
	IC379	" "	
	IC380	" "	
	IC381	" "	
	IC382	" "	
	117	Cattle isolate (Argentina)[d]	+ / +
	126	" "	
	73	Cattle isolate	- / +
	130	(Netherlands)[d]	+ / +
	6	" "	
	47	Human isolate [d]	- / +
	85	" "	- / +
		" "	- / +
M. bovis BCG	Japanese	Vaccine strain [e]	+ / +
	Copenhagen		

a- Dr.Kathleen Eisenach, University of Arkansas, USA
b- Dr. C.N.Paramasivan, Tuberculosis Research Centre, Chennai, India
c- Dr. V.M.Katoch, JALMA, Agra, India
d- Dr. J.D.A. van Embden, Netherlands
e- Department of Paediatrics, AIIMS, New Delhi, India

Table IV: Results of the Direct PCR assay carried out with Bovine Samples

Samples	Detection of Mtb Complex by the PCR Assay		
	Number Tested	Number Positive	Percent
Lymph Gland Biopsy	89	21	23.6
Blood (Heparinsed)	89	01	01.1
Pharyngeal Swab	89	02	02.2
Faeces	89	02	02.2
Rectal Pinch	89	03	03.4
Milk	89	11	12.4
Total Tested	534	40	07.5

The following bovine samples were found to be appropriate for the PCR based assay for detection of bovine tuberculosis: Lymph Gland Biopsy and Milk were found to be the best (Chi square test, p value < 0.05 at significance level, (SAS 8.0, Statistical Software).

Table V : Comparative Analysis Of Clinical & AFB Status of Cattle With Direct PCR Results

Clinical Status		Number Positive For	
Category	Number	Acid Fast Bacilli	PCR
A	17	13	07
B	12	Nil	Nil
C	20	12	09
D	20	05	08
E	20	Nil	02
Total	89	30 (33.7%)	26 (29.2%)

A - Tuberculin Positive with Clinical Signs of Tuberculosis
B - Tuberculin Positive, Apparently healthy Animal
C - Tuberculin Negative with Clinical Signs of Tuberculosis
D - Tuberculin Negative Apparently healthy Animal
E - Animal Infected with non-mycobacterial infection

Among the clinical categories of animals investigated, bovine tuberculosis was detected least in animals infected with non-mycobacterial micro-organisms (Category E), compared to all other categories ($p<0.05$, (Chi square test, p value < 0.05 at significance level, SAS 8.0, Statistical Software).

Table VI: Nested PCR based identification of Pathogenic Mycobacteria in Cattle Derived Samples.

Samples[a]	N- PCR based identification of[b]	
	M.tuberculosis	*M.bovis*
Lymph Gland Biopsy	15	18
Blood[c]	14	14
Milk	26	26
Total Tested 192	55 (28.6%)	58 (30.2%)

a- 64 Samples tested in each category
b- Nested PCR for the C terminal region of the *hup B* gene
c- Citrated Blood

Table VII : Comparative Analysis Of Clinical & AFB Status of Cattle With Nested PCR Results

Clinical Status		*Number Positive For*	
Category	*Number*	*Acid Fast Bacilli*	*N-PCR*
A	20	09	19
B	17	03	16
C	09	03	08
D	10	Nil	07
E	08	Nil	07
Total	64	15 (23.4%)	57 (89.0%)

A - Tuberculin Positive with Clinical Signs of Tuberculosis
B - Tuberculin Positive, Apparently healthy Animal
C - Tuberculin Negative with Clinical Signs of Tuberculosis
D - Tuberculin Negative Apparently healthy Animal
E - Animal Infected with non-mycobacterial infection

Table VIII: Comparison of Bacteriological and PCR-RFLP / Nested - PCR based identification of mycobacterial isolates derived from cattle

Identification of Cattle Derived Mycobacterial Isolates			
Isolate	Classical Criteria	PCR Based Identification	
		M.tuberculosis	*M.bovis*
173	*M.bovis*	+	−
315	*M.tuberculosis*	+	−
262	*M.bovis*	−	+
95	*M.bovis*	−	+
101	*M.bovis*	+	−
113	*M.bovis*	−	+
155	*M.bovis*	−	+
28	*M.bovis*	−	+
36	*M.bovis*	+	−
33	*M.bovis*	−	+

We Claim:

1. Identification of *hupB* gene encoding histone like protein of *M. tuberculosis* and *M. bovis*, a target for detection of *M.tuberculosis* and *M. bovis*.
2. A process for differentiating of the *hupB* gene of *M. tuberculosis* and *M. bovis*.
3. A process as claimed in claim 2 wherein the step of differentiation consists in determining the smaller size of the amplicon obtained from *M. bovis*.
4. A process as claimed in claim 3 wherein the step of determining consists in the detection of PCR amplicon in *M. bovis*.
5. A process as claimed in claim 4 wherein said detection was 27 bp corresponding to 9 amino acids.
6. A process as claimed in claim 4 wherein the detection of PCR amplicon in *M .bovis* was 618 bp.
7. A process as claimed in claim 6 wherein *M. bovis* was 27 bp smaller than that of *M. tuberculosis*.
8. A process as claimed in claim 3, wherein the step of determining comprising designing a set of primers to amplify the C-terminal part of the said gene extracted from *M. tuberculosis* and *M. bovis* to obtain PCR amplified product; analyzing and validating the size of amplicons and sequencing the said PCR product.
9. *hupB* gene as claimed in claim 1 substantially as herein described.
10. A process as in the preceding claims substantially as herein described.

Dated this 26th day of MAY 2003.

(S Bhargava)

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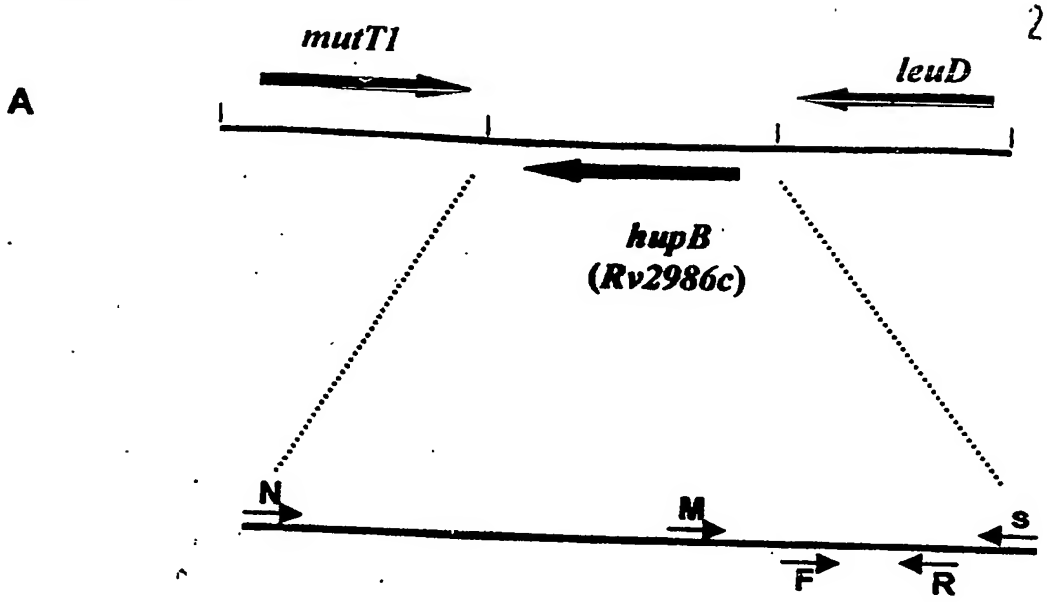
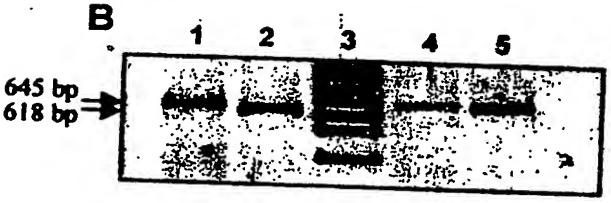
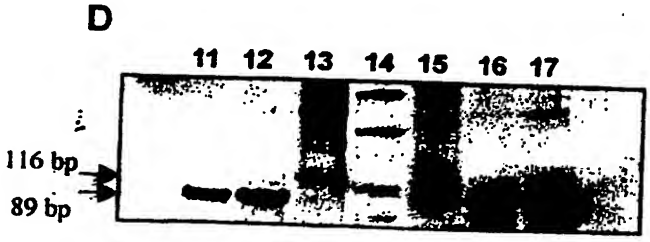




Primers N & S amplification products
- 645 bp in *M. tuberculosis*, (lanes 1 & 4)
- 618 bp in *M. bovis*, (lanes 2 & 5)
- 100 bp Mol. wt. marker (lane 3)

Primers M & S amplification products
- 318 bp in *M. tuberculosis*, (lanes 6 & 10)
- 291 bp in *M. bovis* , (lanes 7 & 9)
- 100 bp Mol. wt . marker (Lane 8)



Primers F & R amplification products
- 116 bp in *M. tuberculosis*, (lanes 13)
- 89 bp in *M. bovis* , (lanes 11,12,15,16,17)
- 100 bp Mol. wt . marker (Lane 14)

Fig : 1

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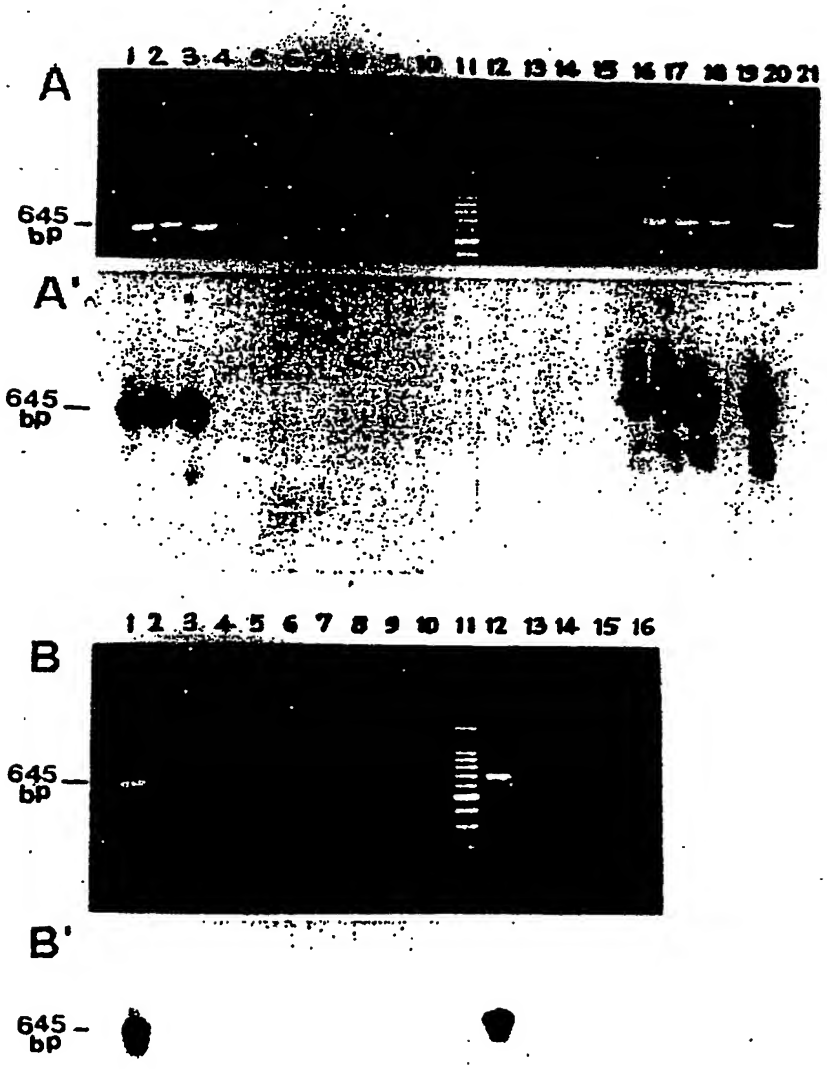

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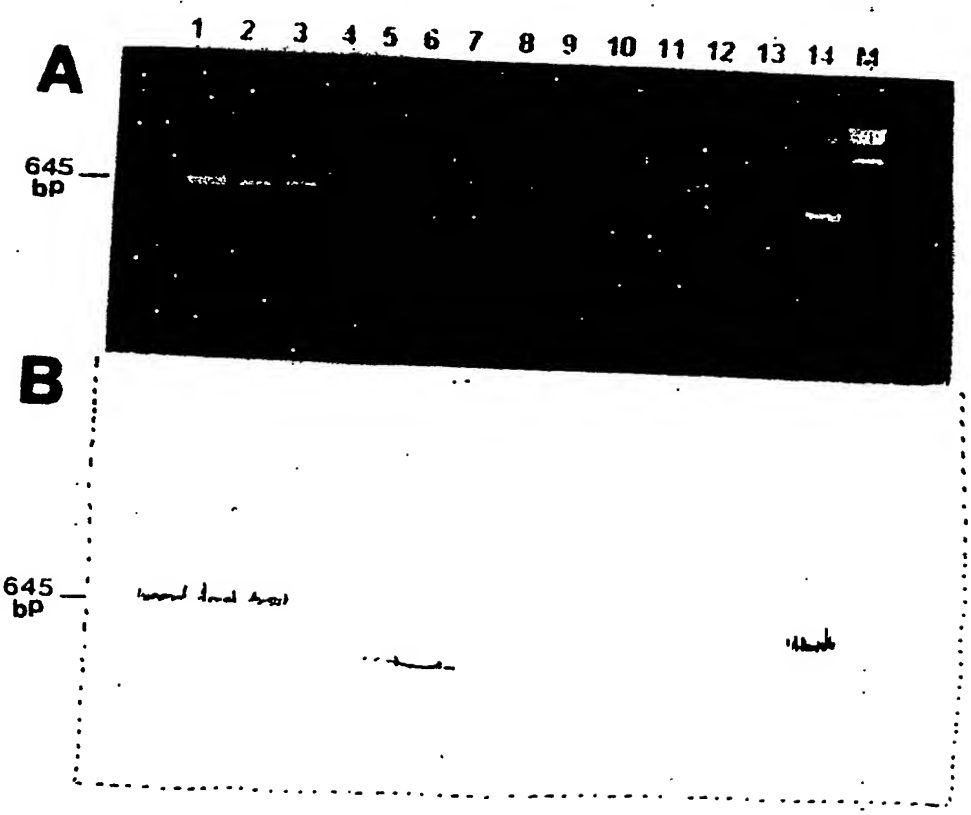

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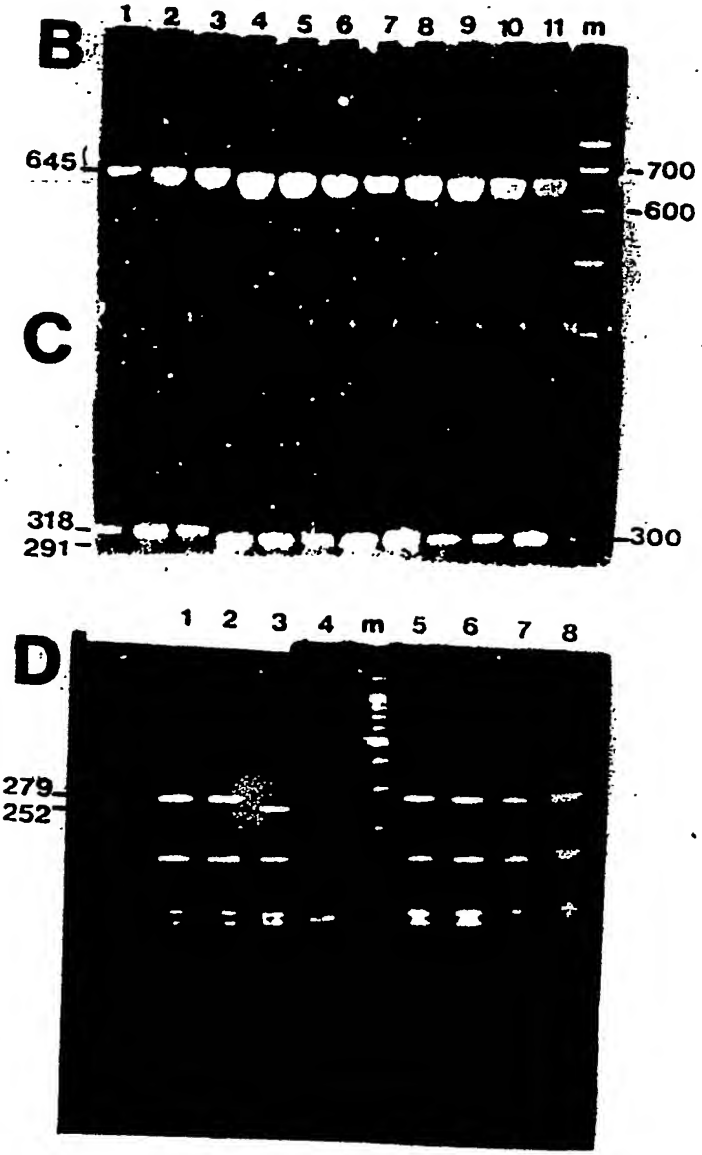

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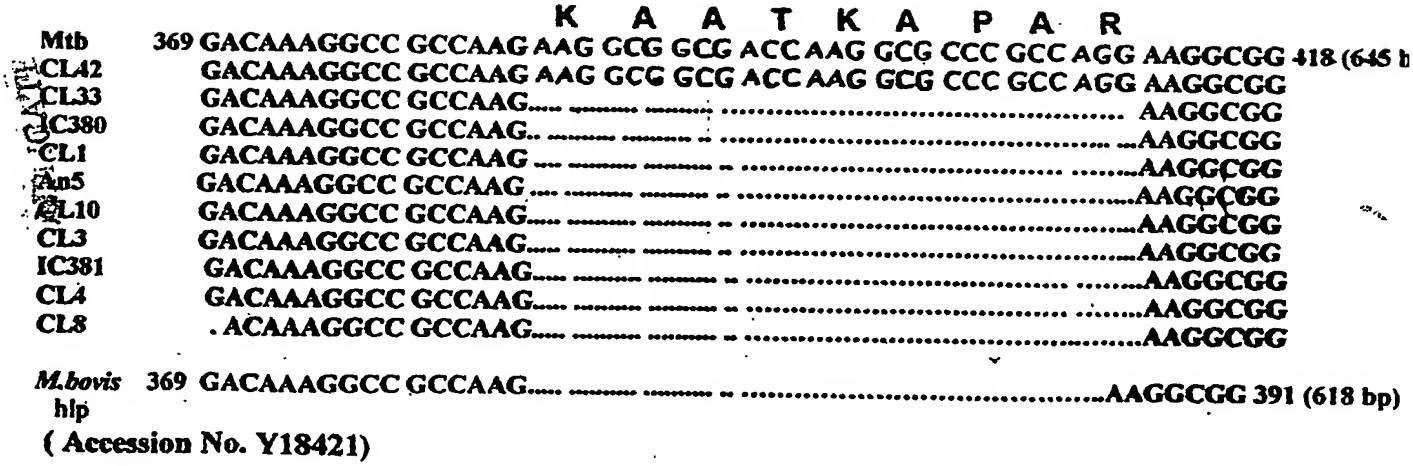

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Fig : 5

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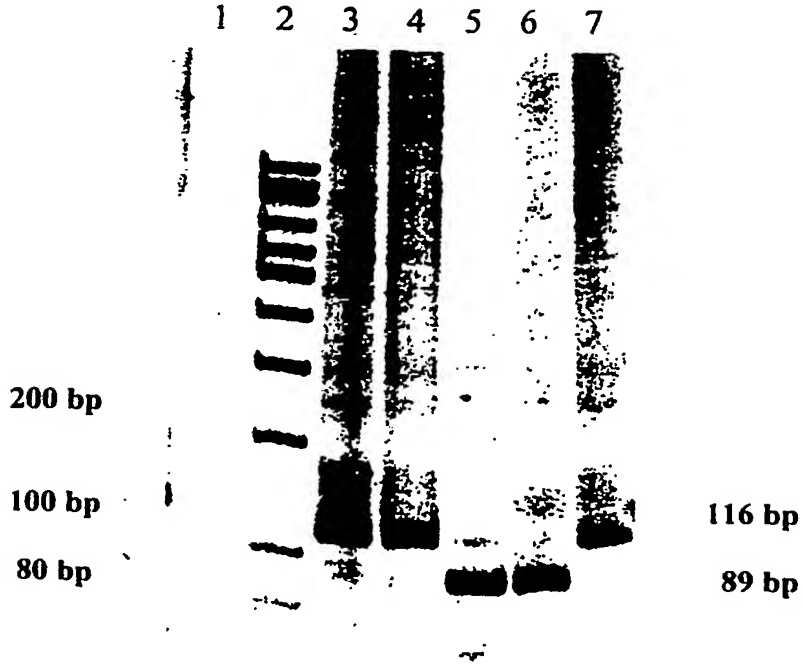

Fig: 6

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